

Guanylate Binding Proteins Enable Rapid Activation of Canonical and Noncanonical Inflammasomes in *Chlamydia*-Infected Macrophages

Ryan Finethy,^a Ine Jorgensen,^b Arun K. Haldar,^a Marcel R. de Zoete,^{c,d} Till Strowig,^{c,e} Richard A. Flavell,^{c,d} Masahiro Yamamoto,^f Uma M. Nagarajan,^g Edward A. Miao,^b Jörn Coers^a

Departments of Molecular Genetics and Microbiology and Immunology, Duke University Medical Center, Durham, North Carolina, USA^a; Department of Microbiology and Immunology and Lineberger Comprehensive Cancer Center, and Center for Gastrointestinal Biology and Disease, University of North Carolina at Chapel Hill, North Carolina, USA^b; Department of Immunobiology, Yale University School of Medicine, New Haven, Connecticut, USA^c; Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, Connecticut, USA^d; Helmholtz Center for Infection Research, Braunschweig, Germany^e; Department of Immunoparasitology, Research Institute for Microbial Diseases, Laboratory of Immunoparasitology, WPI Immunology Frontier Research Center, Osaka University, Yamadaoka, Suita, Osaka, Japan^f; Department of Pediatrics, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA^g

Interferon (IFN)-inducible guanylate binding proteins (GBPs) mediate cell-autonomous host resistance to bacterial pathogens and promote inflammasome activation. The prevailing model postulates that these two GBP-controlled activities are directly linked through GBP-dependent vacuolar lysis. It was proposed that the rupture of pathogen-containing vacuoles (PVs) by GBPs destroyed the microbial refuge and simultaneously contaminated the host cell cytosol with microbial activators of inflammasomes. Here, we demonstrate that GBP-mediated host resistance and GBP-mediated inflammatory responses can be uncoupled. We show that PVs formed by the rodent pathogen *Chlamydia muridarum*, so-called inclusions, remain free of GBPs and that *C. muridarum* is impervious to GBP-mediated restrictions on bacterial growth. Although GBPs neither bind to *C. muridarum* inclusions nor restrict *C. muridarum* growth, we find that GBPs promote inflammasome activation in *C. muridarum*-infected macrophages. We demonstrate that *C. muridarum* infections induce GBP-dependent pyroptosis through both caspase-11-dependent noncanonical and caspase-1-dependent canonical inflammasomes. Among canonical inflammasomes, we find that *C. muridarum* and the human pathogen *Chlamydia trachomatis* activate not only NLRP3 but also AIM2. Our data show that GBPs support fast-kinetics processing and secretion of interleukin-1 β (IL-1 β) and IL-18 by the NLRP3 inflammasome but are dispensable for the secretion of the same cytokines at later times postinfection. Because IFN- γ fails to induce IL-1 β transcription, GBP-dependent fast-kinetics inflammasome activation can drive the preferential processing of constitutively expressed IL-18 in IFN- γ -primed macrophages in the absence of prior Toll-like receptor stimulation. Together, our results reveal that GBPs control the kinetics of inflammasome activation and thereby shape macrophage responses to *Chlamydia* infections.

Gram-negative bacterial species of the genus *Chlamydia* are obligate intracellular pathogens that replicate within the confines of a vacuole referred to as an inclusion. *Chlamydia* species have narrow host ranges and cause a variety of diseases in their respective animal and human hosts (1). Two *Chlamydia* species, *Chlamydia pneumoniae* and *Chlamydia trachomatis*, are human-adapted pathogens. *C. pneumoniae* infects alveolar epithelial cells and macrophages, causing acute respiratory as well as chronic diseases (2). *C. trachomatis* predominantly invades epithelial cells and is responsible for various disease manifestations that depend on the specific bacterial strain and the site of the infection: ocular *C. trachomatis* strains infect conjunctival epithelial cells, resulting in trichiasis, corneal opacification, and blindness; genital *C. trachomatis* strains infect cervical epithelial cells and can cause pelvic inflammatory disease and infertility (3). The estimated number of new cases of sexually transmitted *C. trachomatis* infections amounts to 100 million worldwide per annum, signifying the prevalence of this routinely underreported infection (4).

Because acute *C. trachomatis* infections in women are commonly asymptomatic or cause only mild or nonspecific symptoms, infections often go unnoticed and untreated. A substantial proportion of these untreated infections can progress toward chronic infections that are associated with numerous complications, including chronic pelvic pain, salpingo-oophoritis, fibrosis, and tubal occlusion (3). Host inflammation is believed to be the dominant culprit of these underlying sequelae. This hypothesis is

well supported by numerous studies conducted in mouse models (3). In murine infection models the rodent-adapted species *Chlamydia muridarum* is often used as the infectious agent instead of *C. trachomatis*. *C. muridarum* was first isolated from the lungs of albino Swiss mice that showed flu-like symptoms (5). When instilled into mouse lungs, *C. muridarum* replicates inside alveolar macrophages and causes pneumonia, suggesting that *C. muridarum* is primarily a pulmonary pathogen (6, 7). Subsequently, it was shown that *C. muridarum* could be used to establish genital tract infections that typically resolve within 2 months yet result in oviduct pathologies (8, 9). The incidence and severity of these

Received 30 June 2015 Returned for modification 28 July 2015

Accepted 18 September 2015

Accepted manuscript posted online 28 September 2015

Citation Finethy R, Jorgensen I, Haldar AK, de Zoete MR, Strowig T, Flavell RA, Yamamoto M, Nagarajan UM, Miao EA, Coers J. 2015. Guanylate binding proteins enable rapid activation of canonical and noncanonical inflammasomes in *Chlamydia*-infected macrophages. *Infect Immun* 83:4740–4749. doi:10.1128/IAI.00856-15.

Editor: A. J. Bäuml

Address correspondence to Jörn Coers, jorn.coers@duke.edu.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/IAI.00856-15>.

Copyright © 2015, American Society for Microbiology. All Rights Reserved.

pathologies is diminished in mice concomitantly deficient for two proinflammatory caspases, caspase-1 and caspase-11 (10), suggesting a role for the inflammasome in *Chlamydia*-induced pathologies. Additionally, mice deficient in ASC, a central component of many inflammasomes, develop increased infectious burden and show a delay in the clearance of genital *C. muridarum* infections (11), suggesting a role for inflammasomes in host resistance to *Chlamydia* infections *in vivo*.

Inflammasome complexes typically consist of an upstream sensor protein, the downstream effector proteases caspase-1, caspase-11 (CASP4/5 in humans) or caspase-8, and an adaptor protein, for instance, ASC (12). Inflammasome assembly and activation occur in response to pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). The majority of sensor proteins, including NLRP3 and AIM2, assemble with caspase-1 to form canonical inflammasomes. Once assembled, canonical inflammasomes can process the cytokines interleukin-1 β (IL-1 β) and IL-18 and execute pyroptosis, a type of proinflammatory cell death (12). The proinflammatory caspase-11, on the other hand, is unique in its dual role as both sensor and effector protein of the noncanonical inflammasome. Caspase-11 was shown to bind to lipopolysaccharide (LPS), induce pyroptosis, and activate the NLRP3 inflammasome (13–16). Whereas the importance of caspase-11 in *Chlamydia* pathogenesis was not previously examined, earlier studies showed that *Chlamydia* infections activate the NLRP3 inflammasome (17, 18). Potassium efflux from the cytosol, lysosomal acidification, and cathepsin B release from damaged lysosomes were identified as *Chlamydia* infection-associated patterns that contribute to NLRP3 activation (18). While the aforementioned studies focused on *Chlamydia*-induced responses in unprimed host cells, subsequent studies using LPS-primed macrophages revealed that infections with *Chlamydia* activate inflammasomes other than NLRP3, although the identity of these additional *Chlamydia*-activated inflammasomes was not reported (11).

Macrophage priming augments infection-induced inflammasome responses (12). One class of proteins induced by LPS and other stimuli such as gamma interferon (IFN- γ) are members of the GBP family of IFN-inducible GTPases (19). Recently, it was shown that GBPs promote the lysis of *Salmonella*-containing vacuoles and stimulate caspase-11 activation (20). Because caspase-11 guards cells against Gram-negative bacteria entering the cytosol (21), the execution of pathogen-containing vacuole (PV) lysis was proposed to be the underlying function for GBPs in caspase-11 activation (20). However, our studies on inflammasome activation by *Legionella pneumophila* indicated a role for GBPs in caspase-11 activation that was independent of PV lysis (22). In the current study, we examined the role of GBPs in orchestrating inflammasome-dependent cellular responses to *Chlamydia* infections. We demonstrate that *C. muridarum* inclusions are impervious to GBP binding; therefore, they are resistant to GBP-driven host responses that result in the immediate destruction of the microbe and its vacuolar niche. Although GBP protein family members GBP2 and GBP1 fail to localize to *C. muridarum* inclusions, we show that GBPs promote the induction of pyroptosis through both noncanonical and canonical inflammasomes. We further demonstrate that GBPs promote fast-kinetics but not slow-kinetics inflammasome activation in response to *C. muridarum* infections. Because IFN- γ priming fails to induce IL-1 β expression, the induction of GBP expression by IFN- γ favors the

rapid processing of constitutively expressed IL-18 by NLRP3. These data reveal a unique role for GBPs as accelerators of inflammasome activation in response to *Chlamydia* infections and further show that the kinetics of inflammasome activation can regulate the relative secretion of IL-18 versus IL-1 β , two cytokines with distinct biological activities.

MATERIALS AND METHODS

Cell culture, bacterial strains, infection procedures, and evaluation of bacterial burden. Murine embryonic fibroblasts (MEFs) and Vero cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with nonessential amino acids, 2-mercaptoethanol, and 10% heat-inactivated fetal bovine serum (FBS). To ectopically express GFP-GBP1 fusion protein (where GFP is green fluorescent protein), MEFs were transduced with a previously described retroviral expression vector (23). Macrophages were derived from bone marrow isolated from the indicated mouse lines. Wild-type C57/BL6J mice were purchased from Jackson Laboratories. *GBP^{chr3-/-}* mice harbor a deletion of a cluster of 5 *Gbp* genes located on mouse chromosome 3 and were previously described (24). *Nlrp1^{-/-}*, *Nlrp3^{-/-}*, *Nlr4^{-/-}*, *Aim2^{-/-}*, *Nlrp3^{-/-} Aim2^{-/-}*, *Asc^{-/-}*, *Casp1^{-/-}*, *Casp11^{-/-}*, and *Casp1^{-/-} Casp11^{-/-}* mice were previously described (25–33). BMMs were cultured in RPMI 1640 supplemented with 2-mercaptoethanol, 20% FBS, and 14% conditioned media containing macrophage colony-stimulating factor. *C. muridarum* Nigg and *C. trachomatis* serovar L2 434/Bu were propagated in Vero cells and purified as previously described (34). A previously described GFP expression vector (35) was introduced into LGV-L2 for visualizing *C. trachomatis*. *C. trachomatis* and *C. muridarum* elementary bodies (EBs) were purified by sequential density gradients, as described previously (36). The multiplicity of infection (MOI) for all experiments was defined based on the infectivity of the purified EB stocks in unprimed Vero cells. For infections, *C. muridarum* or *C. trachomatis* was diluted in appropriate culture medium and added to plates at the indicated MOI. To assess bacterial replication in macrophages, BMMs were infected at the relatively low MOI of 3 to minimize MOI-dependent restriction of bacterial growth, as recently reported (37). To monitor the activation of pyroptosis, BMMs were infected at the relatively high MOI of 30. Plates then were centrifuged for 30 min at 1,560 \times g at 10°C in a Sorvall ST 40R centrifuge (Thermo Scientific). The bacterial burden in infected BMMs was determined via quantitative PCR (qPCR) as previously described (38).

Immunocytochemistry and data analysis. Cells were washed with phosphate-buffered saline (PBS), pH 7.4, prior to fixation. Cells were fixed with methanol for 5 min, washed thrice with PBS, and blocked in 5% bovine serum albumin for 30 min at room temperature. *C. muridarum* and *C. trachomatis* inclusions were stained with a mouse monoclonal antibody against *Chlamydia* LPS (used at 1:50; Santa Cruz Biotechnology). A previously described rabbit polyclonal anti-GBP2 antibody (39) was used at 1:1,000. Following incubation with primary antibodies, cells were stained with Alexa Fluor-conjugated secondary antibodies (Molecular Probes) and Hoechst. For colocalization studies, MEFs were infected with *C. muridarum* or *C. trachomatis* at an MOI of 1 and fixed for 24 h postinfection (hpi). The fraction of GBP2-positive inclusions was determined by dividing the number of GBP2-positive inclusions by the total number of counted inclusions. For macrophage studies, cells were infected with *C. muridarum* at an MOI of 3 and fixed 24 hpi. The percentage of inclusion-positive BMMs was determined by dividing the number of BMMs containing an inclusion by the total number of counted macrophages and multiplied by 100. Imaging was performed using a Zeiss Axioskop 2 upright epifluorescence microscope, a Zeiss LSM 510 inverted confocal microscope, or a Leica SP5 inverted confocal microscope.

Cytotoxicity assay and data analysis. BMMs were seeded at a density of 4.0 \times 10⁵ cells per well into 96-well plates. BMMs were primed with IFN- γ (100 U/ml) or LPS (100 ng/ml) or were left unprimed for 16 h. Following priming, BMMs were infected with *C. muridarum* or *C. trachomatis* at an MOI of 30. Cytotoxicity was measured 8 hpi as a function of

relative lactate dehydrogenase (LDH) release using the CytoTox One homogeneous membrane integrity assay (Promega). Relative LDH release was calculated with the following formula: (LDH sample – LDH untreated control)/(LDH lysed control – LDH untreated control) × 100. Measurements were performed on an EnSpire 2300 (PerkinElmer) multilabel reader.

Cytokine measurements. BMMs were seeded at a density of 4.0×10^5 cells per well into 96-well plates. BMMs were primed with IFN- γ (100 U/ml) or LPS (100 ng/ml) or were left unprimed for 6 h. Following priming, BMMs were infected with *C. muridarum* or *C. trachomatis* at an MOI of 30. Supernatants were collected 8 and 24 hpi and stored at -80°C until analysis. IL-1 β and IL-18 were measured by enzyme-linked immunosorbent assay (ELISA; BioLegend/eBioscience). Measurements were performed on an EnSpire 2300 (PerkinElmer) multilabel reader.

Quantitating mRNA levels. BMMs were primed with IFN- γ (100 U/ml) or LPS (100 ng/ml) or were left unprimed for 16 h. Where indicated, BMMs then were infected with *C. muridarum* at an MOI of 30 or 3. RNA from BMMs was isolated following priming or 4 and 8 h postinfection using the RNeasy minikit (Qiagen) by following the manufacturer's instructions. RNA was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad Laboratories), and qPCR was performed with PerfeCTa SYBR green FastMix (Quanta BioSciences) using a StepOnePlus real-time PCR system (Applied Biosystems). Relative mRNA levels were calculated by normalization against transcript levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The following primers were used: mGBP2 F, 5'-CTGCACTATGTGACGGAGCTA-3'; mGBP2 R, 5'-GAGTCCACACAAAGGTTGGAAA-3'; mGBP3 F, 5'-GAGGCACCCATTGTCTGGT-3'; mGBP3 R, 5'-CCGTCTGCAAGACGATTCA-3'; mGBP5 F, 5'-CAGACCTATTTGAACGCCAAAGA-3'; mGBP5 R, 5'-TGCCTTGATTCTATCAGCCTCT-3'; mGBP7 F, 5'-TCCTGTGTGCCTAGTGGAAAA-3'; mGBP7 R, 5'-CAAGCGGTTTCATCAAGTAGGAT-3'; mL-1 β F, 5'-GCAACTGTTCTGAACTCAACT-3'; mL-1 β R, 5'-ATCTTTTGGGGTCCGTCAACT-3'; mL-18 F, 5'-GACTCTTGCGTCAACTCAAGG-3'; mL-18 R, 5'-CAGGCTGTCTTTTGTCACGA-3'; mGAPDH F, 5'-GGTCCTCAGTGTAGCCCAAG-3'; mGAPDH R, 5'-AATGTGTCCGTCGTGGATCT-3'.

Statistical analysis. Where designated, statistical significance was determined using the unpaired Student *t* test or two-way analysis of variance (ANOVA), as appropriate. The level of significance was depicted as $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***)

RESULTS

***Chlamydia muridarum* is resistant to GBP-mediated cell-autonomous immunity in macrophages.** While IFN- γ -induced cell-autonomous immune responses efficiently restrict the growth of the human-adapted pathogen *C. trachomatis* in murine cells, the growth of the closely related rodent-adapted pathogen *C. muridarum* in murine cells is largely unchanged by IFN- γ priming (9, 40). In agreement with these previous observations, we found that IFN- γ priming did not have a significant effect upon *C. muridarum* burden in bone marrow-derived macrophages (BMMs) as assessed by qPCR (Fig. 1A). Likewise, we detected no significant change in the number of inclusion-bearing BMMs at 24 h postinfection (hpi) with IFN- γ priming (Fig. 1B). Because we previously observed that IFN- γ -mediated restriction of *C. trachomatis* in murine cells was dependent on a set of 5 GBPs (GBP1, GBP2, GBP3, GBP5, and GBP7) encoded by mouse chromosome 3 (41), we asked whether the same set of GBPs affected *C. muridarum* burden in IFN- γ -primed BMMs. We found that *GBP^{chr3-/-}* BMMs lacking all 5 *GBP* genes on chromosome 3 were as permissive for *C. muridarum* growth as wild-type BMMs (Fig. 1A). These data suggested that *C. muridarum* escaped from GBP-mediated

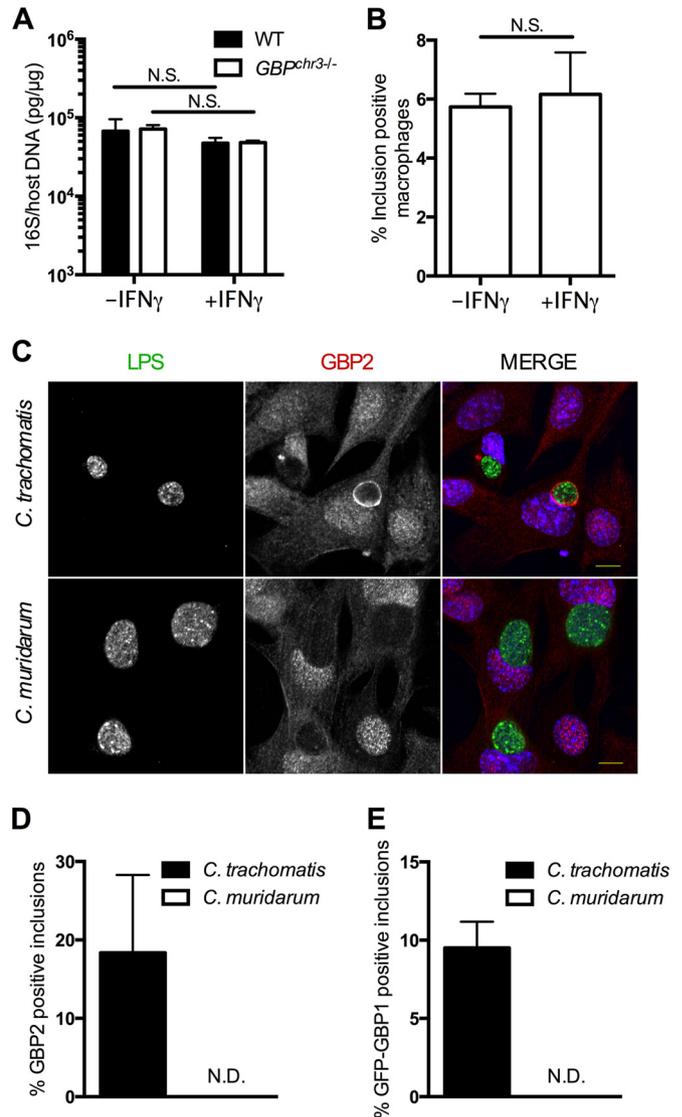


FIG 1 *C. muridarum* resists GBP-mediated cell-autonomous immunity. (A) Wild-type and *GBP^{chr3-/-}* BMMs were primed with 100 U/ml IFN- γ overnight or left unprimed and subsequently infected with *C. muridarum* at an MOI of 3. At 24 hpi, DNA was extracted from infected BMMs and bacterial burden was determined via qPCR. Data are presented as means \pm SEM from 3 independent experiments. Statistical significance was calculated via two-way ANOVA. N.S., not significant. (B) Following infection with *C. muridarum*, wild-type BMMs were fixed at 24 hpi and stained with anti-LPS antibody and Hoechst. The percentage of macrophages containing inclusions with multiple bacteria (>10) was quantified. Data are shown as means \pm SEM from 3 independent experiments. Statistical significance was calculated using Student's *t* test. N.S., not significant. (C and D) IFN- γ -primed MEFs were infected with *C. trachomatis* or *C. muridarum* at an MOI of 1 and stained with Hoechst, anti-*Chlamydia* LPS, and anti-GBP2 antibodies. (D) Frequency of GBP2 localization to inclusions was quantified. (E) MEFs ectopically expressing GFP-GBP1 were primed with IFN- γ and infected with *C. trachomatis* or *C. muridarum* at an MOI of 1. The frequency of GFP-GBP1 localization to inclusions was quantified. Data shown for panels D and E represent means \pm SEM from \sim 800 inclusions and 2 independent experiments. N.D., none detected.

host defense pathways that directly intervene with bacterial replication or survival.

A second set of antimicrobial GTPases involved in cell-autonomous immunity to *Chlamydia* infections is the immunity-re-

lated GTPases (IRGs) (42–44). We previously showed that *C. muridarum* precludes these antimicrobial IRGs from docking to its surrounding inclusion membrane and thereby evades IRG-mediated immune responses (39). Because IRGs control the translocation of GBPs to PVs (45), we hypothesized that *C. muridarum* could block not only IRGs but also GBPs from docking to its inclusion. To test this idea, we infected IFN- γ -primed cells with either *C. trachomatis* or *C. muridarum* and monitored the subcellular localization of GBP2 at 24 hpi. Since we failed to detect any *C. trachomatis* inclusions in IFN- γ -primed BMMs (data not shown), we conducted these experiments in mouse embryonic fibroblasts (MEFs). As previously reported (41, 45), we found that GBP2 decorated *C. trachomatis* inclusions efficiently (Fig. 1C and D). In contrast to the robust targeting of endogenous GBP2 to *C. trachomatis* inclusions, we found that GBP2 was absent from *C. muridarum* inclusions formed either in MEFs (Fig. 1C and D) or in macrophages at various times postinfection (data not shown; also see Fig. S1 in the supplemental material). Similar to endogenous GBP2, ectopically expressed GFP-GBP1 colocalized with *C. trachomatis* but not *C. muridarum* inclusions (Fig. 1E). More detailed studies on the mechanism of GBP binding to *C. trachomatis* inclusion membranes confirmed these observations (46). Together, these data indicated that *C. muridarum* barred GBPs from binding to its surrounding inclusion membrane, an activity likely underlying the observed resistance of *C. muridarum* to GBP-mediated bactericidal or bacteriostatic effects that help eliminate infections with *C. trachomatis*.

GBPs promote pyroptosis in *Chlamydia*-infected, IFN- γ -primed macrophages. We and others previously demonstrated that GBPs promote caspase-11-dependent pyroptosis in response to infections with the Gram-negative bacterial species *L. pneumophila* and *Salmonella enterica* serovar Typhimurium (20, 22). While one study reported that GBPs disrupted PVs, enabling bacteria to enter the host cytosol and activate caspase-11 (20), our data suggested that GBPs acted downstream of PV lysis (22). To test these two models further, we monitored cell death in response to infections with the aforementioned closely related *Chlamydia* species *C. trachomatis* and *C. muridarum*, which fundamentally differ in their permissiveness for GBP binding to their respective inclusions (Fig. 1C to E). We used density gradient-purified *Chlamydia* (EBs) to infect BMMs and found that infections with either GBP-permissive *C. trachomatis* (Fig. 2A) or GBP-resistant *C. muridarum* (Fig. 2B) triggered substantial GBP-dependent cell death in IFN- γ -primed BMMs at 8 hpi. The observed cell death was dependent on inflammatory caspases 1 and 11, indicative of pyroptosis (Fig. 2A and B). These results suggested a role for GBPs in the rapid activation of inflammasomes that was independent of GBP binding to PVs; therefore, it also was independent of GBP-mediated PV lysis.

***C. muridarum* infection triggers GBP-dependent IL-18 secretion in IFN- γ -primed macrophages.** Density gradient-purified *C. muridarum* EBs induced only moderate secretion of either IL-18 or IL-1 β in naive macrophages over the course of 24 hpi (Fig. 3A and B and data not shown), which is in agreement with the general characterization of *Chlamydia* as a stealth pathogen. Since inflammasome activation in naive BMMs was poor, we next asked whether macrophage priming would increase inflammasome-mediated cytokine secretion following *C. muridarum* infections. We found that IFN- γ priming led to a 3-fold increase in IL-18 and a 2-fold increase in IL-1 β secretion in *C. muridarum*-

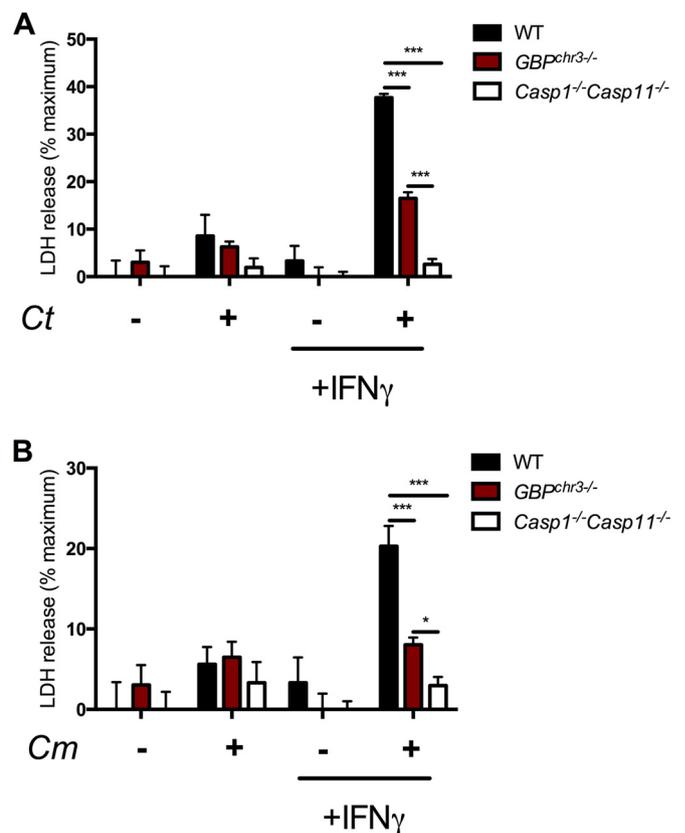


FIG 2 GBPs promote pyroptosis in *Chlamydia*-infected macrophages. Wild-type, *GBP^{chr3-/-}*, and *Casp1^{-/-}Casp11^{-/-}* BMMs were primed with 100 U/ml IFN- γ for 6 h or left unprimed. LDH release from *C. trachomatis*-infected (*Ct*) (A) or *C. muridarum*-infected (*Cm*) (B) BMMs was measured at 8 hpi. Infections were carried out at an MOI of 30. Data are given as means \pm standard deviations (SD) from 4 independent wells. Data are representative of 3 experiments. Statistical significance was calculated using two-way ANOVA (*, $P < 0.05$; ***, $P < 0.001$).

infected wild-type BMMs (Fig. 3A and B). These data suggested that IFN- γ priming promoted inflammasome activation in response to *C. muridarum* infections, possibly through the induction of core inflammasome components or the induction of additional auxiliary host proteins.

Because GBPs were required for the rapid induction of pyroptosis in response to *Chlamydia* infections (Fig. 2A and B), we investigated their possible involvement in IL-1 β or IL-18 secretion. As expected, we observed that *Casp1^{-/-}Casp11^{-/-}* BMMs were deficient for IL-18 or IL-1 β secretion (Fig. 3A and B). We further noticed that GBPs promoted IL-18 secretion but were unexpectedly dispensable for IL-1 β secretion induced by *C. muridarum* infections in IFN- γ -primed BMMs at 24 hpi (Fig. 3A and B). To account for this observation, we considered that GBPs controlled either IL-1 β or IL-18 mRNA expression based on the previous characterization of GBPs as transcriptional regulators of cytokine expression (47). Refuting this hypothesis, we found IL-18 transcript levels to be similar between wild-type and *GBP^{chr3-/-}* BMMs under both primed as well as unprimed conditions and in the presence or absence of an infection (Fig. 3C; also see Fig. S2 in the supplemental material). Although we observed a minor decrease in IL-1 β mRNA levels in *GBP^{chr3-/-}* BMMs (Fig. 3D), the biological relevance of this decrease seemed unclear, since IL-1 β

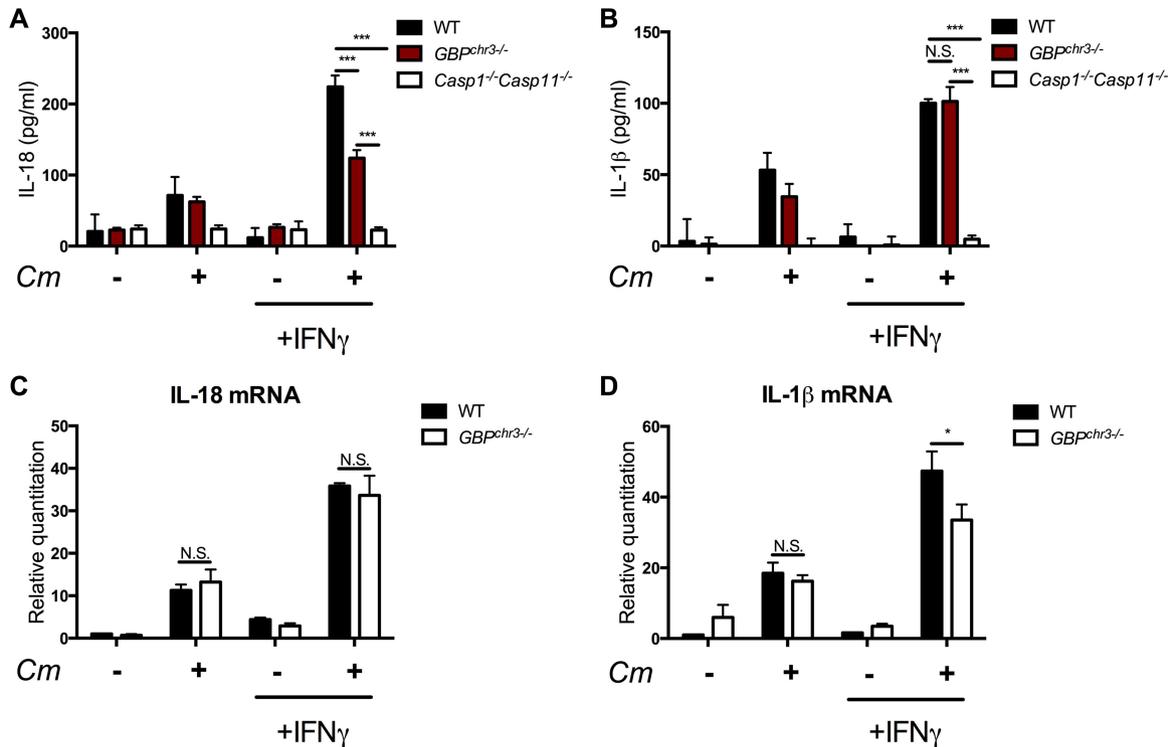


FIG 3 GBPs promote IL-18 but not IL-1 β secretion in IFN- γ -primed BMMs infected with *C. muridarum*. Wild-type, *GBP^{chr3-/-}*, and *Casp1^{-/-}Casp11^{-/-}* BMMs were primed with 100 U/ml IFN- γ or left unprimed. Subsequently, cells were infected with *C. muridarum* at an MOI of 30. IL-18 (A) and IL-1 β (B) concentrations of cell supernatants were determined by ELISA at 24 hpi. Data are displayed as means \pm SD from 4 independent wells and are representative of 3 experiments. (C and D) Wild-type and *GBP^{chr3-/-}* BMMs were primed with 100 U/ml IFN- γ or left unprimed. Cells next were either infected with *C. muridarum* at an MOI of 3 or left uninfected. RNA was extracted at 8 hpi, and relative IL-18 (C) and IL-1 β (D) transcript levels were determined via qPCR. Data are shown as means \pm SEM from 3 independent experiments. Two-way ANOVA was used to calculate statistical significance. *, $P < 0.05$; ***, $P < 0.001$; N.S., not significant.

secretion was unchanged in *GBP^{chr3-/-}* BMMs (Fig. 3B). Therefore, we pursued the alternative hypothesis that GBPs could regulate the kinetics of inflammasome activation and thereby skew the macrophage response toward the preferential processing of constitutively expressed pro-IL-18 available at early times postinfection (48).

GBPs promote fast-kinetics inflammasome activation in response to *C. muridarum* infections. We hypothesized that GBPs could promote inflammasome activation at early times postinfection when constitutively expressed pro-IL-18 but not pro-IL-1 β was available for caspase-1-mediated processing in IFN- γ -primed BMMs. This hypothesis demanded that priming conditions that simultaneously induced the expression of pro-IL-1 β and GBPs render IL-1 β secretion GBP dependent at early times postinfection. One priming agent that induced robust IL-1 β transcription is LPS (Fig. 4A), as previously demonstrated (12). LPS priming also induced the robust expression of GBP2, GBP3, GBP5, and GBP7 (Fig. 4A). We next asked whether IL-1 β secretion in response to *C. muridarum* infections became GBP dependent once BMMs were primed with LPS. In agreement with our hypothesis, we found that LPS but not IFN- γ priming licensed GBP-dependent IL-1 β secretion (Fig. 4B). The secretion of constitutively expressed IL-18, on the other hand, remained GBP dependent regardless of the priming agent (Fig. 4C). In further support for a role for GBPs in regulating inflammasome activation, specifically at early times postinfection, we observed that the quantity

of IL-18 or IL-1 β secreted in the 8- to 24-hpi time window was similar between wild-type and *GBP^{chr3-/-}* BMMs (Fig. 4D and E). These results suggested that only early (between 0 and 8 hpi) but not late (between 8 and 24 hpi) IL-18 and IL-1 β processing required GBPs. Collectively, these observations supported a model according to which GBPs promote inflammasome activation in response to *C. muridarum* infections with fast kinetics but are not involved in inflammasome activation at later times postinfection.

Canonical and noncanonical inflammasomes contribute to *Chlamydia*-induced pyroptosis. To move toward an understanding of the molecular role that GBPs play in controlling cellular responses to *Chlamydia* infections, we set out to identify the specific types of inflammasomes activated by *Chlamydia*. Previously only the canonical NLRP3 inflammasome was reported to be activated by *Chlamydia* infections (11, 17, 18, 49). Here, we found that *C. muridarum* induced robust cell death in IFN- γ -primed wild-type and *Casp1^{-/-}* BMMs at 8 hpi. Dually deficient *Casp1^{-/-}Casp11^{-/-}* BMMs, on the other hand, were resistant to *C. muridarum*-induced cell death, whereas *Casp11^{-/-}* BMMs were moderately protected against cell death (Fig. 5A), demonstrating that *C. muridarum* induces pyroptosis through both canonical and noncanonical inflammasomes. Similarly, pyroptosis induced by *C. trachomatis* infections also was dependent on both caspase-1 and caspase-11 (see Fig. S3 in the supplemental material). Collectively, these data showed that *Chlamydia* infections in

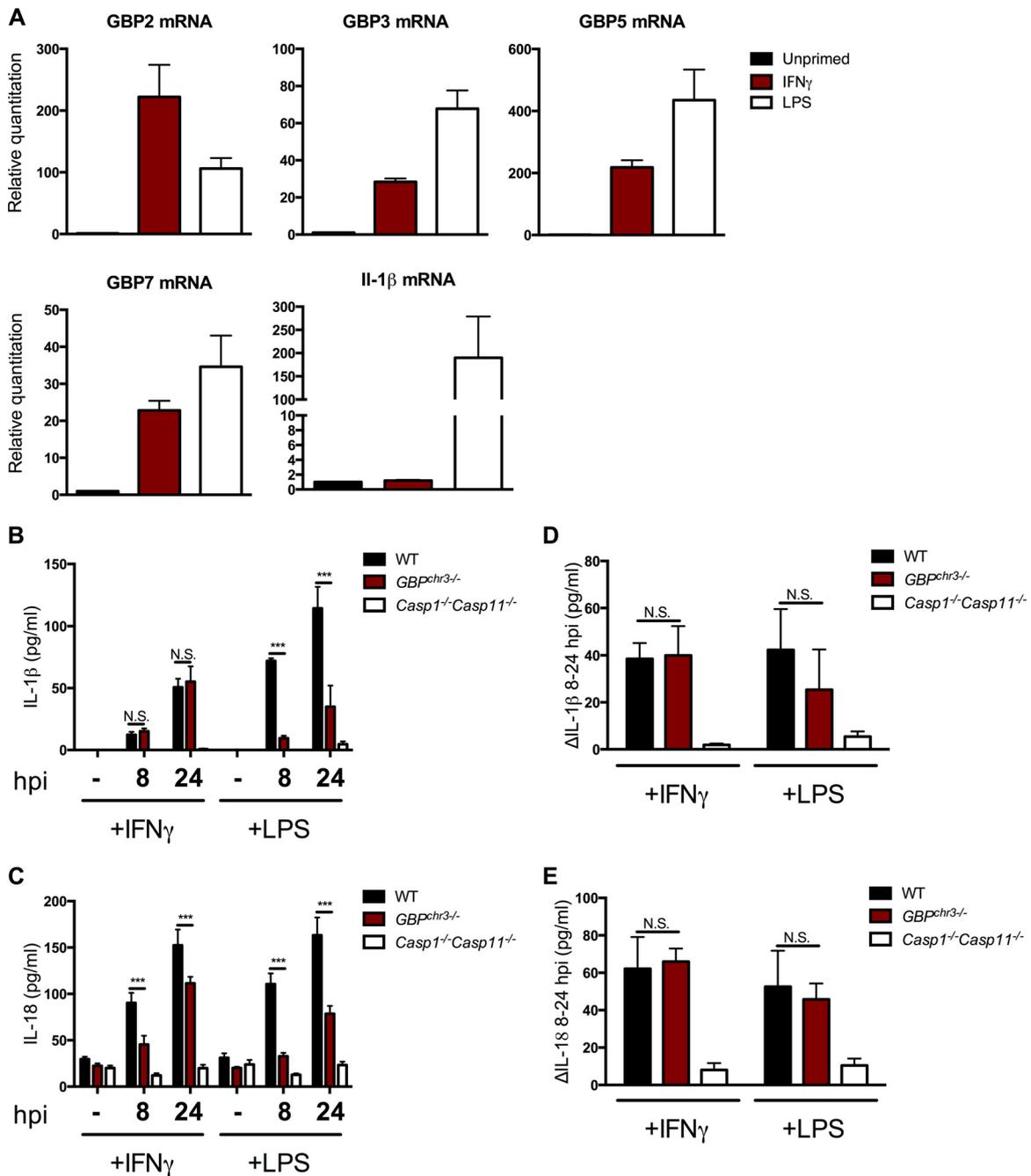


FIG 4 GBPs promote fast-kinetics inflammasome activation. Wild-type BMMs were left unprimed or were primed with either 100 U/ml IFN- γ or 100 ng/ml LPS. (A) Following priming for 6 h, RNA was extracted and GBP2, GBP3, GBP5, GBP7, and IL-1 β mRNA expression was assessed by qPCR. Data are shown as means \pm SEM from 3 independent experiments. (B to E) Wild-type, *GBP^{chr3-/-}*, and *Casp1^{-/-}Casp11^{-/-}* BMMs were primed with 100 U/ml IFN- γ or 100 ng/ml LPS for 6 h and then infected with *C. muridarum* at an MOI of 30. Cell supernatants were collected at 8 and 24 hpi, and IL-1 β (B) and IL-18 (D) concentrations were determined by ELISA. Data are given as means \pm SD from 3 independent wells and represent one of three repeat experiments. Changes in IL-1 β (C) and IL-18 (E) cytokine levels between 8 and 24 hpi were determined by subtracting mean supernatant cytokine levels at 8 hpi from 24-hpi data. Data are shown as means \pm SD from 3 independent wells. N.S., not significant; ***, $P < 0.001$ (based on two-way ANOVA).

BMMs activated both canonical and noncanonical inflammasomes at 8 hpi. Because the induction of pyroptosis at 8 hpi in *Chlamydia*-infected BMMs was GBP dependent (Fig. 2), these results strongly argued that GBPs regulated both canonical and non-canonical inflammasome activation in response to *Chlamydia* infections.

AIM2 and NLRP3 are required for IL-1 β and IL-18 secretion in *Chlamydia*-infected macrophages. While both caspase-1 and caspase-11 were required for the execution of pyroptosis, we found that only caspase-1 expression was essential for the secretion of IL-1 β and IL-18 following *C. muridarum* infections (Fig. 5B and C). These results demonstrated that *C. muridarum* infec-

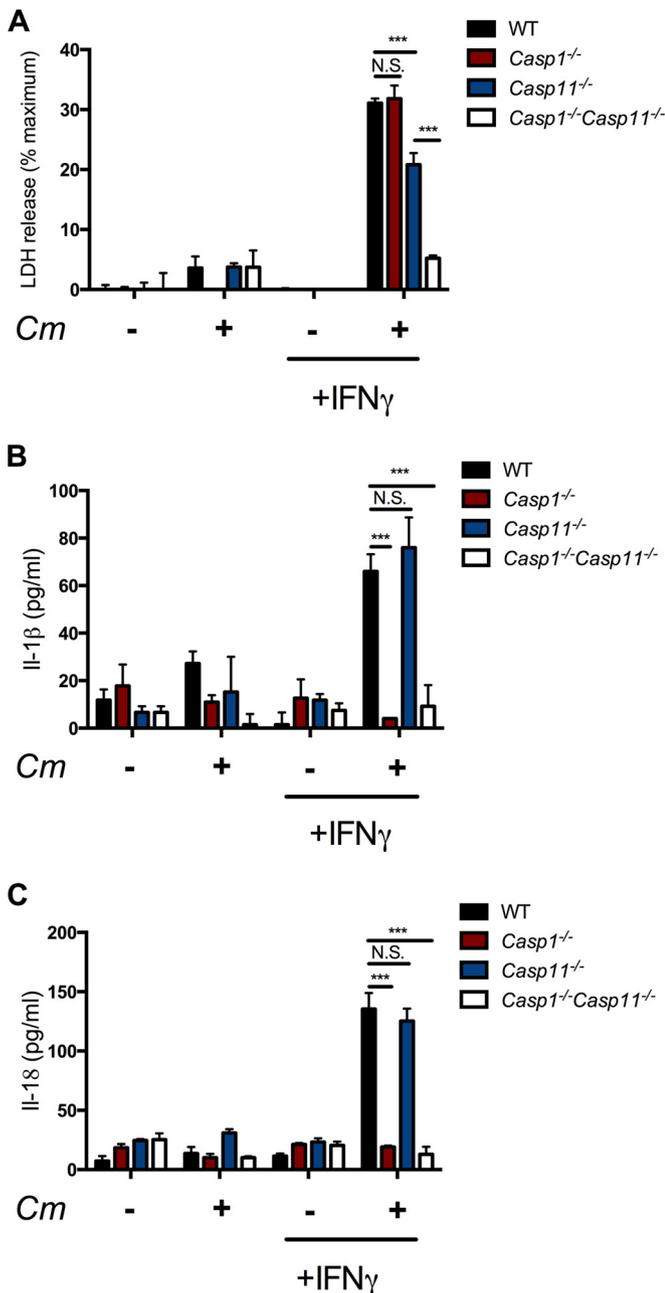


FIG 5 *C. muridarum* infections activate canonical and noncanonical inflammasomes. Wild-type, *Casp1*^{-/-}, *Casp11*^{-/-}, and *Casp1*^{-/-} *Casp11*^{-/-} BMMs were primed with 100 U/ml IFN- γ or left unprimed and subsequently infected with *C. muridarum* at an MOI of 30. (A) LDH release was measured at 8 hpi. IL-1 β (B) and IL-18 (C) cytokine concentrations in cell supernatants were measured via ELISA at 24 hpi. Data are shown as means \pm SD from 3 independent wells. Data are representative of 3 independent experiments. N.S., not significant; ***, $P < 0.001$ (based on two-way ANOVA).

tions induced IL-1 β and IL-18 secretion independently of caspase-11 activation. To identify the *Chlamydia*-induced canonical inflammasome activation pathway, we set out to define the repertoire of caspase-1-dependent canonical inflammasomes responsible for *Chlamydia*-induced IL-1 β and IL-18 processing and secretion. In accordance with previous reports (11, 17, 18, 49), we found that *Chlamydia*-infected *Nlrp3*^{-/-} BMMs secreted less

IL-1 β and IL-18 than wild-type BMMs did (Fig. 6; also see Fig. S4 and S5 in the supplemental material). However, IFN- γ -primed *Nlrp3*^{-/-} BMMs infected with *C. muridarum* secreted significantly more IL-18 than caspase-1-deficient BMMs did at 24 hpi (see Fig. S4), indicating that *C. muridarum* infections activate at least one NLRP3-independent canonical inflammasome. To identify the additional *C. muridarum*-induced canonical inflammasome, we monitored IL-18 secretion in IFN- γ -primed and *C. muridarum*-infected *Nlrp1*^{-/-}, *Nlrp3*^{-/-}, *Nlrp4*^{-/-}, *Aim2*^{-/-}, *Asc*^{-/-}, and *Casp1*^{-/-} *Casp11*^{-/-} BMMs (see Fig. S4). We observed a complete defect in IL-18 secretion in *Asc*^{-/-} and *Casp1*^{-/-} *Casp11*^{-/-} BMMs, a partial reduction in IL-18 secretion in *Nlrp3*^{-/-} and *Aim2*^{-/-} BMMs, and no change in IL-18 secretion in *Nlrp1*^{-/-} and *Nlrp4*^{-/-} BMMs relative to that of wild-type BMMs (see Fig. S4), implying a role for AIM2 in the cellular response to *C. muridarum* infections. In further support of *C. muridarum*-triggered AIM2 activation, we found that dually deficient *Nlrp3*^{-/-} *Aim2*^{-/-} BMMs primed with IFN- γ or LPS failed to secrete IL-1 β or IL-18 in response to *C. muridarum* infections, whereas *Nlrp3*^{-/-} BMMs displayed a partial defect at 24 hpi (Fig. 6A and B). Infections with *C. trachomatis* also induced both AIM2- and NLRP3-dependent cellular responses at 24 hpi (see Fig. S5), demonstrating that AIM2- and NLRP3-containing inflammasomes mediate IL-1 β and IL-18 secretion in response to *Chlamydia* infections.

Fast induction of IL-18 secretion in IFN- γ -primed macrophages requires NLRP3 but not AIM2. Lastly, we asked whether fast-kinetics inflammasome activation in response to *C. muridarum* infections was dependent on NLRP3 or AIM2 by monitoring cytokine secretion at 8 hpi. We observed that IL-18 secretion at 8 hpi in IFN- γ -primed BMMs was dependent solely on NLRP3 (Fig. 6B). Because IL-18 secretion is partially dependent on GBPs under these experimental conditions (Fig. 4C), these data indicated that GBPs specifically promoted the activation of the NLRP3 inflammasome, as previously reported (50). Unexpectedly, we noticed a moderate but reproducible increase in IL-18 and IL-1 β secretion in IFN- γ -primed *Aim2*^{-/-} BMMs that we failed to observe under LPS priming conditions (Fig. 6A and B). Instead, AIM2 played a more prominent role in IL-18 and IL-1 β secretion in LPS-primed BMMs, suggesting that LPS and IFN- γ differentially modulate AIM2 and NLRP3 activities by an unknown mechanism. Regardless of the priming stimulus, we found that AIM2 and NLRP3 both contributed to IL-18 and IL-1 β secretion at 24 hpi (Fig. 6A and B). While a role for GBPs in *Chlamydia*-induced AIM2 activation could not be excluded at this time, our observations strongly suggested that GBPs controlled the rapid activation of the NLRP3 inflammasomes in *C. muridarum*-infected BMMs.

DISCUSSION

IFN- γ -inducible GBPs provide cell-autonomous resistance to viral, bacterial, and protozoan pathogens (19, 23, 24, 41, 51–53). More recently, GBPs were implicated as cellular cofactors required for canonical and noncanonical inflammasome activation in response to bacterial infections (20, 22, 50, 54–56). While all of these recent studies showed that GBPs augmented cellular responses associated with inflammasome activation, they differed substantially in the proposed modes of action by which GBPs regulate inflammasomes (20, 50, 54, 55). One matter of contention revolves around the question of whether or not GBPs need to bind to and lyse PVs in order to activate inflammasomes (20, 22,

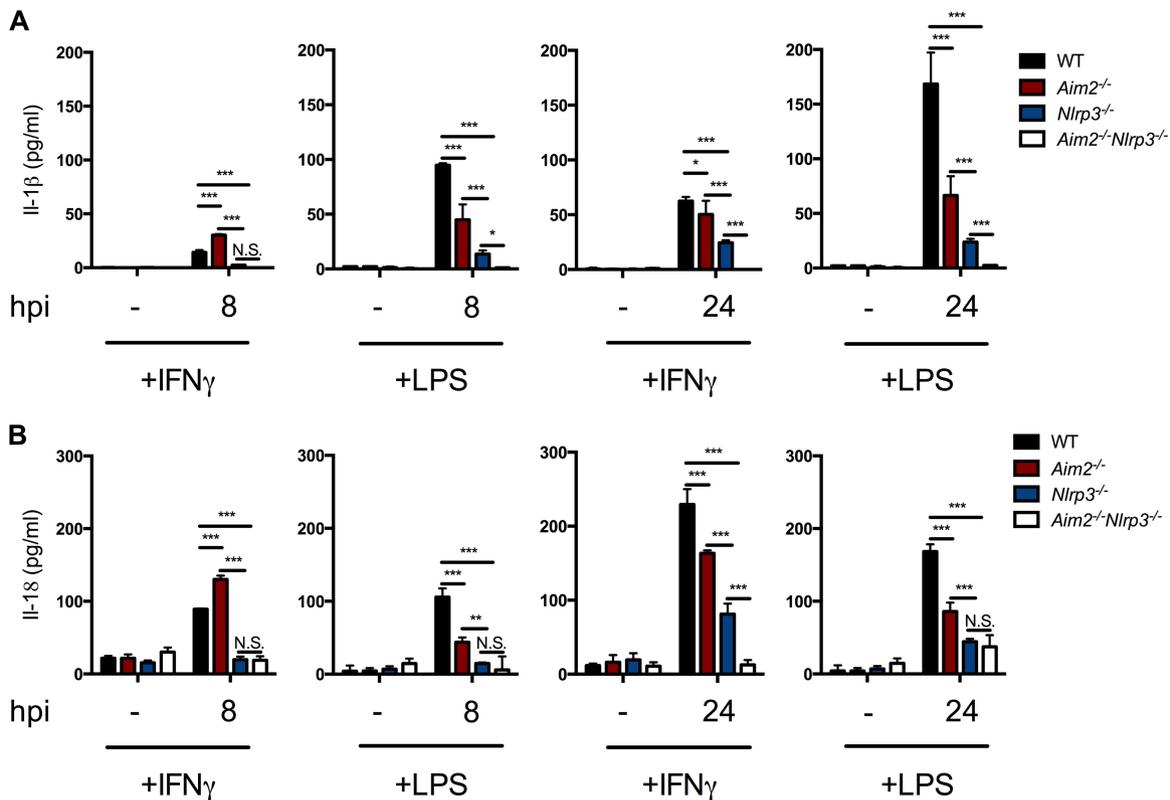


FIG 6 Differential activation of AIM2 and NLRP3 inflammasomes by *C. muridarum* in IFN- γ - and LPS-primed macrophages. Wild-type, *Aim2*^{-/-}, *Nlrp3*^{-/-}, and *Aim2*^{-/-}*Nlrp3*^{-/-} BMMs were primed with 100 U/ml IFN- γ or 100 ng/ml LPS. Cells were infected with *C. muridarum* and supernatants collected at 8 or 24 hpi. Concentrations of IL-1 β (A) and IL-18 (B) in cell supernatants were measured by ELISA. Data are depicted as means \pm SD from 3 independent wells and are representative of 2 experiments. Statistical significance was calculated using two-way ANOVA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; N.S., not significant.

50). Here, we identified *C. muridarum* as a pathogen that evades GBP binding to its surrounding vacuole yet still activates inflammasomes in a partially GBP-dependent manner. While we do not necessarily dismiss a role for GBPs in PV lysis, our observations argue that GBPs play an important role in inflammasome activation by a vacuolar pathogen that is independent of PV lysis.

At least three models have been put forward to account for the role of GBPs in inflammasome activation. For vacuolar pathogens it was proposed that GBPs mediate PV lysis, thereby allowing for the spillage of PAMPs into the host cytosol (20). In support of this model, GBPs were previously shown to promote the destruction of parasitophorous vacuoles formed by *Toxoplasma gondii* (24). However, our current observations in a *C. muridarum* infection model and our previous studies on *L. pneumophila* (22) failed to identify a link between GBPs and PV lysis. These observations made in distinct infection models are not necessarily contradictory to one another and could be explained if both GBP-dependent and GBP-independent PV lysis pathways existed that are of varying importance depending on the pathogen or experimental conditions used. Circumstantial evidence supports the existence of GBP-independent PV lysis pathways: we previously found that Galectin-3, a marker of disrupted vacuoles, colocalizes with *Legionella*-containing vacuoles in *GBP*^{chr3-/-} BMMs (22); here, we observed that *C. muridarum* establishes productive inclusions in only a fraction of infected BMMs regardless of the *GBP* genotype (Fig. 1 and data not shown), while dispersed chlamydial LPS is found in the majority of both wild-type and *GBP*^{chr3-/-} BMMs

(data not shown). Whether or not the GBP-independent loss of vacuolar integrity observed for *Legionella*-containing vacuoles and *Chlamydia* inclusions is the result of host-directed PV lysis will require further investigation.

In addition to PV lysis, GBPs also have been characterized as mediators of direct bacteriolysis. Recent studies demonstrated that GBPs were required for inflammasome activation in response to infections with *Francisella novicida*, a pathogen that actively egresses from its phagosome into the host cytosol (54, 55). As it was shown that GBPs bind to cytosolic *F. novicida*, it seems plausible that GBPs also could bind to and lyse PV-resident pathogens, such as *L. pneumophila* or *Chlamydia*, once these pathogens have been expelled from their respective vacuolar niches. While this hypothesis is attractive and deserves further testing, we have failed to observe any association of cytosolically expelled *Chlamydia* and GBP proteins (data not shown). GBP-dependent bacteriolysis also would be expected to release bacterial DNA, thereby triggering robust AIM2 activation. However, the data presented here and in previous studies suggest that GBPs promote predominantly caspase-11 and NLRP3 inflammasome activation in response to infections with the vacuolar pathogens *C. muridarum*, *S. Typhimurium*, and *L. pneumophila* (20, 22, 50). Therefore, we favor a model in which GBPs directly promote caspase-11 and NLRP3 inflammasome activation. Our model is in agreement with a previous report demonstrating that at least one member of the GBP family, i.e., GBP5, can complex with the inflammasome sensor

protein NLRP3 and can alter its activity (50), although this assertion has been challenged (20, 54).

As already alluded to, a third mechanism of action by which GBPs control inflammasome responses was proposed, according to which at least one GBP family member promotes NLRP3-ASC oligomerization. It was shown that tetrameric GBP5 promotes NLRP3-dependent ASC oligomerization, potentially through the clustering of PYD domains (50). These observations raised several important questions, one of which pertained to the nature of the stimulus that would induce GBP5 tetramerization. We propose that infectious agents, which include *Chlamydia* species, release unidentified PAMPs or DAMPs that trigger tetramerization of GBP5 and potentially tetramerization of other GBPs controlling inflammasome function. A second question prompted by these previous studies concerns whether additional GBPs other than GBP5 physically and functionally interact with inflammasome components. While our studies were conducted in BMMs lacking 5 GBP-encoding genes, including *GBP5*, future studies will address which specific GBPs augment inflammasome activation in response to *Chlamydia* infections.

Here, we also made the unexpected observation that GBPs were specifically required for fast-kinetics inflammasome activation that occurred within the first 8 h after *C. muridarum* infection. We observed that rapid GBP-dependent inflammasome activation was specifically induced by viable, metabolically active *C. muridarum* (data not shown), alluding to the possibility that an event occurring during the initial stage of the *Chlamydia* infectious cycle triggers GBP-dependent responses. Alternatively, the relatively low abundance of inflammasome stimuli during the initial phase of the infection may require GBPs as high-affinity amplifiers, a requirement that expires at later stages of the infection. The discovery of GBP-interacting molecules most likely will shed more light on the mechanism by which GBPs accelerates inflammasome activation and reveal why GBPs are uniquely required for fast-kinetics inflammasome activation in *C. muridarum*-infected cells.

Interestingly, we observed that changes in the kinetics of canonical inflammasome activation could alter the quality of the macrophage response to *C. muridarum* infections: because IFN- γ induces the expression of GBPs but not IL-1 β , GBP-driven rapid inflammasome activation in IFN- γ -primed macrophages results in the preferential processing of constitutively expressed IL-18. The immunological activities of IL-1 β and IL-18 are distinct and sometimes antagonistic (48, 57). Therefore, GBP-dependent alterations in the relative secretion of IL-1 β versus IL-18 could impact the immune response to *Chlamydia* and other pathogens that trigger GBP-dependent inflammasome pathways during a Th1-biased immune response. Therefore, our future studies will address whether GBPs control inflammation-associated pathologies in the context of infections with *Chlamydia* and other pathogens and whether therapeutic intervention targeting GBPs can alter the course and outcome of inflammation.

ACKNOWLEDGMENTS

We thank Thirumala-Devi Kanneganti for sharing *NLRP3*^{-/-} *AIM2*^{-/-} mouse bone marrow cells.

This work was supported by grant 15H01377 for Scientific Research on Innovative Areas from MEXT (to M.Y.), a National Science Foundation predoctoral award (to R.F.), and by National Institute Health grant R01AI103197 (to J.C.).

REFERENCES

- Belland R, Ojcius DM, Byrne GI. 2004. Chlamydia. *Nat Rev Microbiol* 2:530–531.
- Roulis E, Polkinghorne A, Timms P. 2013. Chlamydia pneumoniae: modern insights into an ancient pathogen. *Trends Microbiol* 21:120–128.
- Hafner LM. 2015. Pathogenesis of fallopian tube damage caused by Chlamydia trachomatis infections. *Contraception* 92:108–115. <http://dx.doi.org/10.1016/j.contraception.2015.01.004>.
- World Health Organization. 2011. Global prevalence and incidence of selected curable sexually transmitted diseases: overview and estimates. World Health Organization, Geneva, Switzerland.
- Nigg C. 1942. An unidentified virus which produces pneumonia and systemic infection in mice. *Science* 95:49–50.
- Kuo C, Chen WJ. 1980. A mouse model of Chlamydia trachomatis pneumonitis. *J Infect Dis* 141:198–202.
- Williams DM, Schachter J, Coalson JJ, Grubbs B. 1984. Cellular immunity to the mouse pneumonitis agent. *J Infect Dis* 149:630–639.
- Swenson CE, Donegan E, Schachter J. 1983. Chlamydia trachomatis-induced salpingitis in mice. *J Infect Dis* 148:1101–1107.
- Perry LL, Su H, Feilzer K, Messer R, Hughes S, Whitmire W, Caldwell HD. 1999. Differential sensitivity of distinct *Chlamydia trachomatis* isolates to IFN-gamma-mediated inhibition. *J Immunol* 162:3541–3548.
- Cheng W, Shivshankar P, Li Z, Chen L, Yeh IT, Zhong G. 2008. Caspase-1 contributes to Chlamydia trachomatis-induced upper urogenital tract inflammatory pathologies without affecting the course of infection. *Infect Immun* 76:515–522.
- Nagarajan UM, Sikes JD, Yeruva L, Prantner D. 2012. Significant role of IL-1 signaling, but limited role of inflammasome activation, in oviduct pathology during *Chlamydia muridarum* genital infection. *J Immunol* 188:2866–2875.
- Latz E, Xiao TS, Stutz A. 2013. Activation and regulation of the inflammasomes. *Nat Rev Immunol* 13:397–411.
- Rathinam VA, Vanaja SK, Waggoner L, Sokolovska A, Becker C, Stuart LM, Leong JM, Fitzgerald KA. 2012. TRIF licenses caspase-11-dependent NLRP3 inflammasome activation by gram-negative bacteria. *Cell* 150:606–619.
- Hagar JA, Powell DA, Aachoui Y, Ernst RK, Miao EA. 2013. Cytoplasmic LPS activates caspase-11: implications in TLR4-independent endotoxic shock. *Science* 341:1250–1253.
- Kayagaki N, Wong MT, Stowe IB, Ramani SR, Gonzalez LC, Akashi-Takamura S, Miyake K, Zhang J, Lee WP, Muszynski A, Forsberg LS, Carlson RW, Dixit VM. 2013. Noncanonical inflammasome activation by intracellular LPS independent of TLR4. *Science* 341:1246–1249.
- Shi J, Zhao Y, Wang Y, Gao W, Ding J, Li P, Hu L, Shao F. 2014. Inflammatory caspases are innate immune receptors for intracellular LPS. *Nature* 514:187–192. <http://dx.doi.org/10.1038/nature13683>.
- Abdul-Sater AA, Said-Sadier N, Padilla EV, Ojcius DM. 2010. Chlamydial infection of monocytes stimulates IL-1 β secretion through activation of the NLRP3 inflammasome. *Microbes Infect* 12:652–661.
- He X, Mekasha S, Mavrogiorgos N, Fitzgerald KA, Lien E, Ingalls RR. 2010. Inflammation and fibrosis during *Chlamydia pneumoniae* infection is regulated by IL-1 and the NLRP3/ASC inflammasome. *J Immunol* 184:5743–5754.
- Degrandi D, Konermann C, Beuter-Gunia C, Kresse A, Wurthner J, Kurig S, Beer S, Pfeffer K. 2007. Extensive characterization of IFN-induced GTPases mGBP1 to mGBP10 involved in host defense. *J Immunol* 179:7729–7740.
- Meunier E, Dick MS, Dreier RF, Schurmann N, Kenzelmann Broz D, Warming S, Roose-Girma M, Bumann D, Kayagaki N, Takeda K, Yamamoto M, Broz P. 2014. Caspase-11 activation requires lysis of pathogen-containing vacuoles by IFN-induced GTPases. *Nature* 509:366–370.
- Aachoui Y, Leaf IA, Hagar JA, Fontana MF, Campos CG, Zak DE, Tan MH, Cotter PA, Vance RE, Aderem A, Miao EA. 2013. Caspase-11 protects against bacteria that escape the vacuole. *Science* 339:975–978.
- Pilla DM, Hagar JA, Haldar AK, Mason AK, Degrandi D, Pfeffer K, Ernst RK, Yamamoto M, Miao EA, Coers J. 2014. Guanylate binding proteins promote caspase-11-dependent pyroptosis in response to cytoplasmic LPS. *Proc Natl Acad Sci U S A* 111:6046–6051.
- Virreira Winter S, Niedelmann W, Jensen KD, Rosowski EE, Julien L, Spooner E, Caradonna K, Burleigh BA, Saeij JP, Ploegh HL, Frickel EM.

2011. Determinants of GBP recruitment to *Toxoplasma gondii* vacuoles and the parasitic factors that control it. *PLoS One* 6:e24434.
24. Yamamoto M, Okuyama M, Ma JS, Kimura T, Kamiyama N, Saiga H, Ohshima J, Sasai M, Kayama H, Okamoto T, Huang DC, Soldati-Favre D, Horie K, Takeda J, Takeda K. 2012. A cluster of interferon-gamma-inducible p65 GTPases plays a critical role in host defense against *Toxoplasma gondii*. *Immunity* 37:302–313. <http://dx.doi.org/10.1016/j.immuni.2012.06.009>.
 25. Case CL, Kohler LJ, Lima JB, Strowig T, de Zoete MR, Flavell RA, Zamboni DS, Roy CR. 2013. Caspase-11 stimulates rapid flagellin-independent pyroptosis in response to *Legionella pneumophila*. *Proc Natl Acad Sci U S A* 110:1851–1856.
 26. Jones JW, Kayagaki N, Broz P, Henry T, Newton K, O'Rourke K, Chan S, Dong J, Qu Y, Roose-Girma M, Dixit VM, Monack DM. 2010. Absent in melanoma 2 is required for innate immune recognition of *Francisella tularensis*. *Proc Natl Acad Sci U S A* 107:9771–9776.
 27. Kanneganti TD, Ozoren N, Body-Malapel M, Amer A, Park JH, Franchi L, Whitfield J, Barchet W, Colonna M, Vandenabeele P, Bertin J, Coyle A, Grant EP, Akira S, Nunez G. 2006. Bacterial RNA and small antiviral compounds activate caspase-1 through cryopyrin/Nalp3. *Nature* 440:233–236.
 28. Karki R, Man SM, Malireddi RK, Gurung P, Vogel P, Lamkanfi M, Kanneganti TD. 2015. Concerted activation of the AIM2 and NLRP3 inflammasomes orchestrates host protection against *Aspergillus* infection. *Cell Host Microbe* 17:357–368.
 29. Kayagaki N, Warming S, Lamkanfi M, Vande Walle L, Louie S, Dong J, Newton K, Qu Y, Liu J, Heldens S, Zhang J, Lee WP, Roose-Girma M, Dixit VM. 2011. Non-canonical inflammasome activation targets caspase-11. *Nature* 479:117–121.
 30. Kovarova M, Hesker PR, Jania L, Nguyen M, Snouwaert JN, Xiang Z, Lommatzsch SE, Huang MT, Ting JP, Koller BH. 2012. NLRP1-dependent pyroptosis leads to acute lung injury and morbidity in mice. *J Immunol* 189:2006–2016.
 31. Kuida K, Lippke JA, Ku G, Harding MW, Livingston DJ, Su MS, Flavell RA. 1995. Altered cytokine export and apoptosis in mice deficient in interleukin-1 beta converting enzyme. *Science* 267:2000–2003.
 32. Mariathasan S, Newton K, Monack DM, Vucic D, French DM, Lee WP, Roose-Girma M, Erickson S, Dixit VM. 2004. Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature* 430:213–218.
 33. Sutterwala FS, Ogura Y, Szczepanik M, Lara-Tejero M, Lichtenberger GS, Grant EP, Bertin J, Coyle AJ, Galan JE, Askenase PW, Flavell RA. 2006. Critical role for NALP3/CIAS1/Cryopyrin in innate and adaptive immunity through its regulation of caspase-1. *Immunity* 24:317–327.
 34. Coers J, Gondek DC, Olive AJ, Rohlfing A, Taylor GA, Starnbach MN. 2011. Compensatory T cell responses in IRG-deficient mice prevent sustained *Chlamydia trachomatis* infections. *PLoS Pathog* 7:e1001346.
 35. Wang Y, Kahane S, Cutcliffe LT, Skilton RJ, Lambden PR, Clarke IN. 2011. Development of a transformation system for *Chlamydia trachomatis*: restoration of glycogen biosynthesis by acquisition of a plasmid shuttle vector. *PLoS Pathog* 7:e1002258.
 36. Saka HA, Thompson JW, Chen YS, Kumar Y, Dubois LG, Moseley MA, Valdivia RH. 2011. Quantitative proteomics reveals metabolic and pathogenic properties of *Chlamydia trachomatis* developmental forms. *Mol Microbiol* 82:1185–1203.
 37. Rajaram K, Nelson DE. 2015. *Chlamydia muridarum* infection of macrophages elicits bactericidal nitric oxide production via reactive oxygen species and cathepsin B. *Infect Immun* 83:3164–3175.
 38. Bernstein-Hanley I, Balsara ZR, Ulmer W, Coers J, Starnbach MN, Dietrich WF. 2006. Genetic analysis of susceptibility to *Chlamydia trachomatis* in mouse. *Genes Immun* 7:122–129.
 39. Coers J, Bernstein-Hanley I, Grotzky D, Parvanova I, Howard JC, Taylor GA, Dietrich WF, Starnbach MN. 2008. *Chlamydia muridarum* evades growth restriction by the IFN-gamma-inducible host resistance factor Irgb10. *J Immunol* 180:6237–6245.
 40. Roshick C, Wood H, Caldwell HD, McClarty G. 2006. Comparison of gamma interferon-mediated antichlamydial defense mechanisms in human and mouse cells. *Infect Immun* 74:225–238.
 41. Haldar AK, Piro AS, Pilla DM, Yamamoto M, Coers J. 2014. The E2-like conjugation enzyme Atg3 promotes binding of IRG and Gbp proteins to *Chlamydia*- and *Toxoplasma*-containing vacuoles and host resistance. *PLoS One* 9:e86684.
 42. Al-Zeer MA, Al-Younes HM, Braun PR, Zerrahn J, Meyer TF. 2009. IFN-gamma-inducible Irga6 mediates host resistance against *Chlamydia trachomatis* via autophagy. *PLoS One* 4:e4588.
 43. Bernstein-Hanley I, Coers J, Balsara ZR, Taylor GA, Starnbach MN, Dietrich WF. 2006. The p47 GTPases Igtg and Irgb10 map to the *Chlamydia trachomatis* susceptibility locus Ctrg-3 and mediate cellular resistance in mice. *Proc Natl Acad Sci U S A* 103:14092–14097.
 44. Nelson DE, Virok DP, Wood H, Roshick C, Johnson RM, Whitmire WM, Crane DD, Steele-Mortimer O, Kari L, McClarty G, Caldwell HD. 2005. Chlamydial IFN-gamma immune evasion is linked to host infection tropism. *Proc Natl Acad Sci U S A* 102:10658–10663.
 45. Haldar AK, Saka HA, Piro AS, Dunn JD, Henry SC, Taylor GA, Frickel EM, Valdivia RH, Coers J. 2013. IRG and GBP host resistance factors target aberrant, “non-self” vacuoles characterized by the missing of “self” IRGM proteins. *PLoS Pathog* 9:e1003414.
 46. Haldar AK, Foltz C, Finethy R, Piro AS, Feeley EM, Pilla-Moffett DM, Komatsu M, Frickel EM, Coers J. 28 September 2015. Ubiquitin systems mark pathogen-containing vacuoles as targets for host defense by guanylate binding proteins. *Proc Natl Acad Sci U S A*
 47. Hu Y, Wang J, Yang B, Zheng N, Qin M, Ji Y, Lin G, Tian L, Wu X, Wu L, Sun B. 2011. Guanylate binding protein 4 negatively regulates virus-induced type I IFN and antiviral response by targeting IFN regulatory factor 7. *J Immunol* 187:6456–6462.
 48. Sedimbi SK, Hagglof T, Karlsson MC. 2013. IL-18 in inflammatory and autoimmune disease. *Cell Mol Life Sci* 70:4795–4808.
 49. Abdoul-Sater AA, Koo E, Hacker G, Ojcius DM. 2009. Inflammasome-dependent caspase-1 activation in cervical epithelial cells stimulates growth of the intracellular pathogen *Chlamydia trachomatis*. *J Biol Chem* 284:26789–26796.
 50. Shenoy AR, Wellington DA, Kumar P, Kassa H, Booth CJ, Cresswell P, MacMicking JD. 2012. GBP5 promotes NLRP3 inflammasome assembly and immunity in mammals. *Science* 336:481–485.
 51. Anderson SL, Carton JM, Lou J, Xing L, Rubin BY. 1999. Interferon-induced guanylate binding protein-1 (GBP-1) mediates an antiviral effect against vesicular stomatitis virus and encephalomyocarditis virus. *Virology* 256:8–14.
 52. Neumann B, Walter T, Heriche JK, Bulkescher J, Erfle H, Conrad C, Rogers P, Poser I, Held M, Liebel U, Cetin C, Sieckmann F, Pau G, Kabbe R, Wunsche A, Satagopam V, Schmitz MH, Chapuis C, Gerlich DW, Schneider R, Eils R, Huber W, Peters JM, Hyman AA, Durbin R, Pepperkok R, Ellenberg J. 2010. Phenotypic profiling of the human genome by time-lapse microscopy reveals cell division genes. *Nature* 464:721–727.
 53. Kim BH, Shenoy AR, Kumar P, Das R, Tiwari S, MacMicking JD. 2011. A family of IFN-gamma-inducible 65-kD GTPases protects against bacterial infection. *Science* 332:717–721.
 54. Man SM, Karki R, Malireddi RK, Neale G, Vogel P, Yamamoto M, Lamkanfi M, Kanneganti TD. 2015. The transcription factor IRF1 and guanylate-binding proteins target activation of the AIM2 inflammasome by *Francisella* infection. *Nat Immunol* 16:467–475. <http://dx.doi.org/10.1038/ni.3118>.
 55. Meunier E, Wallet P, Dreier RF, Costanzo S, Anton L, Ruhl S, Dussurgey S, Dick MS, Kistner A, Rigard M, Degrandi D, Pfeffer K, Yamamoto M, Henry T, Broz P. 2015. Guanylate-binding proteins promote activation of the AIM2 inflammasome during infection with *Francisella novicida*. *Nat Immunol* 16:476–484. <http://dx.doi.org/10.1038/ni.3119>.
 56. Rupper AC, Cardelli JA. 2008. Induction of guanylate binding protein 5 by gamma interferon increases susceptibility to *Salmonella enterica* serovar Typhimurium-induced pyroptosis in RAW 264.7 cells. *Infect Immun* 76:2304–2315.
 57. Garland C, Dinarello CA, Mantovani A. 2013. The interleukin-1 family: back to the future. *Immunity* 39:1003–1018.